BIODEGRADATION OF ALPHA TNT AND ITS PRODUCTION ISOMERS

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Gram negative bacteria have been isolated from several sources which utilize 2, 4,6-trinitrotoluene (α-TNT) as the sole source of carbon and nitrogen for growth. There is a direct relationship between the α-TNT concentration in the growth medium and growth response of isolate I-2WT as well as the percentage of TNT degradation by this organism. Studies using ring UL-^{14}C-TNT suggested possible ring cleavage (biodegradation) of TNT as ^{14}C activity was released as carbon dioxide and also found in the cells. Heterotrophic carbon dioxide fixation was demonstrated using $NaH^{14}CO_3$ added to a culture growing on unlabeled TNT.

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BIODEGRADATION OF ALPHA TNT

AND

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Annual Report-U

by:

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Contract No. DAAG17-73-C-0276

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U. S. Army Natick Development Center
Natick, Massachusetts

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I. Statement of the Problem

The waste products from the manufacture and shell loading of munitions represents a serious disposal problem for the Department of Defense. In the manufacture of TNT, the plant wash water contains quantities (50-100 ppm) of TNT; while the waste from the Sellite Process contains TNT and the production isomers of TNT.

It has been shown that these nitrobodies can be absorbed into an XAD2 column and eluted from the XAD2 column with acetone (now in critical supply) or other organic solvents. This treatment may be an effective solution to this waste disposal problem in that it will generate a nitrobody free effluent water. The XAD2 system is not destructive, therefore, it has not solved the problem of nitrobody disposal, but results in a concentrated waste which may be burned unless explosive dangers or serious air contamination problems are associated with the burning process. If these nitrobodies are found to be biodegradable, they could be rendered ecologically safe by biological treatment.

II. Background

Recalcitrant molecules have specific structural characteristics which prevent microbial metabolism of the molecules, but at this time microbiologists are not able to describe all of these structural characteristics. It can be stated that naturally occurring organic molecules are biodegradable by some microbial form whereas the truly recalcitrant molecules are found in broad classes of the synthetic organic compounds. In nature, many compounds which are potentially biodegradable, are slowly degradable or totally undegradable because of their interaction in the soil or the influence of environmental factors.

In studies of nitrophenols, Raymond and Alexander (1) isolated a bacterium able to metabolize p-nitrophenol and showed the appearance of stoichiometric amounts of nitrite in the medium and the formation of catechol. Tewfik and Evans (2) demonstrated the biodegradation of 3,5-dinitro-o-cresol via reduction of the 3-nitro group to an amino group which is then replaced by a hydroxyl group. Ring fission occurs after the formation of the trihydroxyl species. These observations clearly indicate that substitution of nitro-groups to the benzene ring does not necessarily confer recalcitrance to the molecule.

One of the first serious studies of TNT biodegradation by microorganism was reported by Osmon and Klausmeier (3). In their study they tested for degradation on agar containing 1% yeast ext. and 1% glucose plus 100 mg/liter of TNT. Their measurement of degradation was the loss of TNT from the medium which can only be considered as presumptive evidence of degradation. In no case were they able to detect the disappearance of TNT in flasks containing only mineral salts and TNT.

II. Background (continued)

A recent study by Won, Heckly, Glover and Hoffsommer (4) describes metabolic disposition of TNT. Again they describe the disappearance of TNT but have also included in their media readily oxidizable substrate such as glucose (0.1%), and yeast extract (0.5%). They have demonstrated oxygen uptake by organism suspensions in the presence of TNT. Analysis of culture filtrates shows the conversion of TNT to 2,2',6,6'-tetranitro-4,4'-azoxytoluene; 2,2',4,4'-tetranitro-6,6'-azoxytoluene; 4-amino-2,6-dinitrotoluene; 2-amino-4-hydroxylaminotoluene; and 2-amino-4,6-dinitrotoluene. None of these intermediates indicate biodegradation of the TNT molecule, but only biotransformation of the ring substituted nitrogen groups.

A review of the literature demonstrates that none of the other laboratories working on TNT degradation have yet isolated an organism that will grow in a mineral salts medium with TNT present as the sole source of organic carbon and energy.

III. Materials and Methods

A. Enrichment culture techniques.

Inocula for enrichment cultures have been obtained from Narragansett Bay sediments, University of Rhode Island boiler plant effluent and raw sewage obtained at the inlet pipe of the University of Rhode Island treatment plant. The basal media used for enrichment isolations are described in Table 1.

For aerobic isolations either M-9 or M-9(-N) were used as enrichment media. In the anaerobic isolations, T (7) medium was used as it provided nitrate as an electron acceptor.

Table 1. Mineral Salts Basal Media

Component	M-9*	M-9(-N)	T**
NH_4Cl (g)	1.0		
Na_2HPO_4 (g)	6.0	6.0	1
KH_2PO_4 (g)	3.0	3.0	0.5
NaCl (g)	5.0	5.0	
$MgSO_4$ (g)	0.1	0.1	0.5
NH_4NO_3 (g)			2.5
$CaCl_2$ (mg)			20.0
$FeSO_4$ (mg)			1.0
$MnSO_4$ (mg)			0.01
$Co(NO_3)_2$			0.005
$(NH_4)_6Mo_7O_{24}$			0.1
Distilled water (ml)	1000	1000	1000

*Reference 5
**Reference 7

In all isolations 50 ml of medium containing 100 µg/ml of TNT was used in a 250 ml Erlenmyer flask or a 300 ml Bellco side-arm flask. These were inoculated with 0.1 g of sediment or 1.0 ml of liquid and incubated at 25, 30 and 35°C on gyrorotary shakers at 125 rpm. The primary enrichment cultures were incubated until visible turbidity developed, then 0.1 ml of the primary culture was transferred to a fresh flask of the same medium. When turbidity developed in the secondary enrichment flask, the bacterial population was streaked for isolation onto mineral salts-TNT medium solidified with 1.5% washed agar and the same medium supplemented with 0.01% Difco yeast extract. When indicated 0.01% yeast extract was included in the primary and secondary enrichment flasks.

The pure cultures obtained by this isolation scheme were inoculated at 10^3-10^4 viable cells per ml (80-100 Klett Units) into mineral salts broth containing 100 µg/ml of TNT with or without 0.01% yeast extract supplement. The growth response in this test flask was followed by turbidity measurement in the Klett-Summerson photo-electric colorimeter using the No. 42 blue filter.

Anaerobic isolations have used only T medium, and all incubations were carried out in a National Appliance Company, Model 3640 anaerobic incubator using N_2 as the inert replacement gas.

Enrichments from sewage and the initial sewage degradation studies were done in a continuous flow fermentor. The fermentor vessel was charged with 250 ml of double strength M-9 medium containing 100 µg/ml of TNT and 250 ml of raw sewage. Aeration was set at 500 cc/minute and the incubation temperature was 30°C. This mixture was allowed to incubate for 2 hours, then an M-9 + 100 µg/ml TNT solution was fed through the fermentor vessel at a rate of 2 ml/minute. At intervals, 0.1 ml of 10^{-3}, 10^{-4} and 10^{-5} dilutions from the fermentor vessel were spread plated onto M-9 TNT washed agar with and without 0.01% yeast extract supplement. Representative of each colony type developing on the isolation plates were selected and their growth response on TNT determined in liquid medium.

B. Analytical techniques.

Studies were performed on a gas chromatographic method for the quantitation of TNT but discontinued as we did not feel the values had good reproducibility. We have adopted a method using absorbance at 224 nm in the Carey model 14 spectrophotometer. A standard curve was prepared from the O.D. readings of varying concentrations of alpha-TNT at 224 nm. The standard curve was prepared with known concentrations of TNT in petroleum ether and also with known concentrations of TNT in mineral salts medium, extracted into petroleum ether.

Residual TNT in culture media has been determined by comparison to the standard curve after different treatments of the culture. The bacterial cells were removed by centrifugation, then the clarified supernatant was extracted twice with 25 ml of petroleum ether. The combined extracts were placed in a 100 ml volumetric flask and diluted to 100 ml with petroleum ether. Further dilutions of this extract were made as required to obtain readings within the range of the standard curve.

A second method of quantification has involved lysis of the cells by treating 25 ml of the culture with 5 ml of concentrated H_2SO_4. After lysis, the same extraction procedure was used to determine residual TNT. The lysis procedure was designed to release any cell-bound TNT.

Thin layer chromatography has been used for the detection of TNT and its related nitro-aromatic compounds. The support used in all these experiments was Eastman No. 13179, 20X20 cm Silica Gel sheets without fluorescent indicator.

Developing Solvents:

a. Chloroform, Methanol, Glac. Acetic Acid, 80:20:1

b. Benzene, Hexane, Acetone, 50:50:10

Indicator Sprays:

a. 0.5% Fast Black in distilled water followed by 0.1 N NaOH

b. Ethylenediamine

IV. Results

A. Aerobic enrichments:

A total of 57 aerobic enrichment cultures (Table 2) have been examined for their ability to utilize TNT. Of these 57 isolates, only 19 isolates have passed the first proof step which was increased turbidity upon inoculation into M-9 or modified M-9 medium with TNT as the sole source of carbon. In some cases the cultures passing the first proof step were mixed cultures which, when purified, did not show a positive growth response. At this time, advanced studies have used isolate A25-7-B-X-II (simplified code IIBX) and isolate A7WASN-1 (simplified code I-2).

B. Anaerobic enrichments:

Repeated attempts at the isolation of TNT utilizing organisms under anaerobic conditions from various inocula have failed to yield any isolates of interest to this study.

C. Growth studies with isolate IIBX:

Figure 1 shows the typical growth response of isolate IIBX in M-9 medium containing 100 µg/ml of TNT and 0.01% (100 µg/ml) yeast extract as a supplement. If yeast extract was not present in the medium, there was no turbidity increase beyond the inoculum value. This plot demonstrates that 0.01% (100 µg/ml) of yeast extract will not support growth of isolate IIBX.

Further evidence for the stimulatory nature of yeast extract is provided in Table 3. Yeast extract at even 100 µg/ml does not allow growth of the test organism, but in the presence of 100 µg/ml of TNT provides significantly better growth than lower concentrations of this supplement with TNT.

Table 2. Enrichment isolates, by source and substrate(s).

Inoculum Source	Carbon Source(s)	Culture Number	Isolation Stage	Proof and Lab Number
Garden Soil	TNT	A25-7-G-X	PE	No
Garden Soil	TNT, Yeast Extract	A25-7-G-YE	PE	No
Garden Soil	TNT, Citric Acid	A25-7-G-CA	SE	No
Garden Soil	TNT, Citric Acid, Yeast Extract	A25-7-G-CAYE	SE	No
Garden Soil	TNT, Tartrate	A-25-7-G-NaT	PE	No
Garden Soil	TNT, Succinate	A-25-7-G-SA	PE	No
Garden Soil	TNT, Succinate, Yeast Extract	A-25-7-G-SAYE	PE	No
Garden Soil	TNT, Malonate	A-25-7-G-MA	PE	No
Garden Soil	TNT, Malonate Yeast Extract	A-25-7-G-MAYE	PE	No
Bay Sediment	TNT	A25-7-B-X	PE	Yes-mixed
Bay Sediment	TNT, Yeast Extract	A25-7-B-YE	PE	Yes-mixed
Bay Sediment	TNT, Succinate	A25-7-B-SA	SE	No
Bay Sediment	TNT, Succinate, Yeast Extract	A25-7-B-SAYE	SE	No
Bay Sediment	TNT, Citric Acid	A25-7-B-CA	SE	No
Bay Sediment	TNT, Citric Acid, Yeast Extract	A27-7-B-CAYE	SE	No
Bay Sediment	TNT	A25-7-B-X-I	SE	No
Bay Sediment	TNT	A25-7-B-X-II	SE	Yes IIBX

Table 2. continued

Inoculum Source	Carbon Source(s)	Culture Number	Isolation Stage	Proof and Lab Number
Bay Sediment	TNT	A25-7-B-X-III	SE	No
Bay Sediment	TNT, Yeast Extract	A25-7-B-YE-I	SE	No
Bay Sediment	TNT, Yeast Extract	A25-7-B-YE-II	SE	No
Bay Sediment	TNT, Yeast Extract	A25-7-B-YE-III	SE	No
Bay Sediment	TNT, Yeast Extract	A25-7-B-YE-IV	SE	No
Bay Sediment	TNT	A-1-25	PE	No
Bay Sediment	TNT	b-1-25	PE	No
Bay Sediment	TNT	C-1-25	PE	No
Bay Sediment	TNT	D-1-25	PE	No
Bay Sediment	TNT	A-1-35	PE	No
Bay Sediment	TNT	B-1-35	PE	No
Bay Sediment	TNT	C-1-35	PE	No
Bay Sediment	TNT	D-1-35	PE	No
Soil	TNT, Yeast Extract	25-4-S-II	SE	No
Soil	TNT, Yeast Extract	25-1-S-I	SE	No
Soil	TNT, Yeast Extract	25-1-S	SE	No
Boiler Effluent	TNT, Yeast Extract	25-3-S	SE	No
Boiler Effluent	TNT	35-3-S-N-1	SE	Yes-mixed
Boiler Effluent	TNT	35-3-S-N-2	SE	Yes-mixed
Boiler Effluent	TNT	35-3-S-N-1	SE	No
Boiler Effluent	TNT	35-3-S-III-2	SE	No
Boiler Effluent	TNT, Yeast Extract	A7MS1A	SE	Yes-mixed

Table 2. continued

Inoculum Source	Carbon Source(s)	Culture Number	Isolation Stage	Proof and Lab Number
Boiler Effluent	TNT, Yeast Extract	A7MS1B	SE	Yes-mixed
Boiler Effluent	TNT, Yeast Extract	A7MS2B	SE	Yes-mixed
Boiler Effluent	TNT, Yeast Extract	A7WASL3-1	SE	Yes*
Boiler Effluent	TNT, Yeast Extract	A7WASN-1	SE	Yes* I-2WT
Boiler Effluent	TNT, Yeast Extract	A7WASL3-2	SE	Yes*
Boiler Effluent	TNT, Yeast Extract	A7WASAgIII-1	SE	Yes*
Sewage	TNT	S-9-UY-UN-5	C	Yes*
Sewage	TNT	S-9-UY-UN-12	C	Yes*
Sewage	TNT	S-9-UY-UN-DFW	C	Yes*
Sewage	TNT, Yeast Extract	S-9-SY-UN-1	C	Yes*
Sewage	TNT, Yeast Extract	S-9-SY-UN-2	C	Yes*
Sewage	TNT, Yeast Extract	S-9-SY-UN-3	C	Yes*
Sewage	TNT, Yeast Extract	S-9-SY-UN-4	C	Yes*
Sewage	TNT, Yeast Extract	S-9-SY-UN-5	C	Yes*-mixed
Sewage	TNT, Yeast Extract	S-9-SY-UN-6	C	Yes*
Sewage	TNT, Yeast Extract	S-9-SY-UN-7	C	Not tested*
Sewage	TNT, Yeast Extract	S-9-SY-UN-8	C	Not tested*
Sewage	TNT, Yeast Extract	S-9-SY-UN-9	C	Not tested*

PE = from primary enrichment flask

SE = from secondary enrichment flask

C = from continuous fermentor

* = carried in stock culture

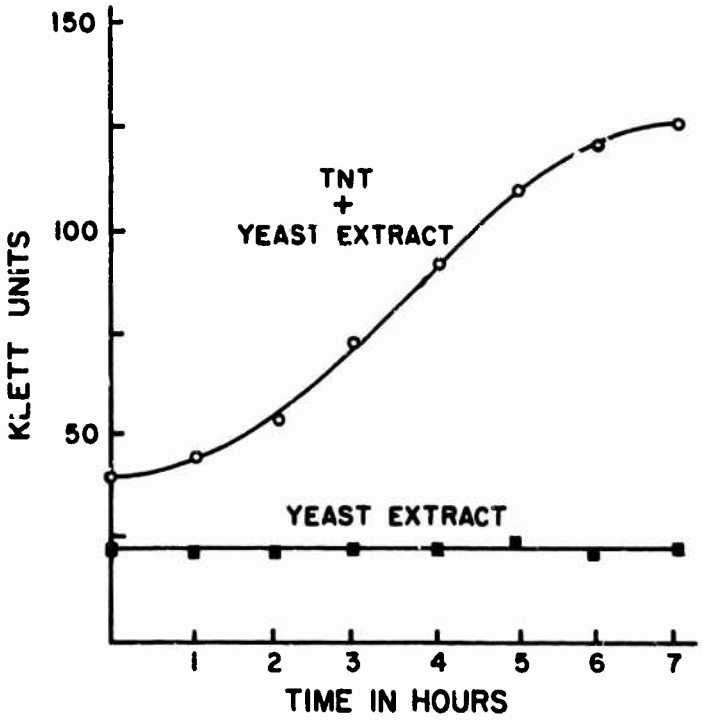

Figure 1. Growth of IIBX on TNT and yeast extract.

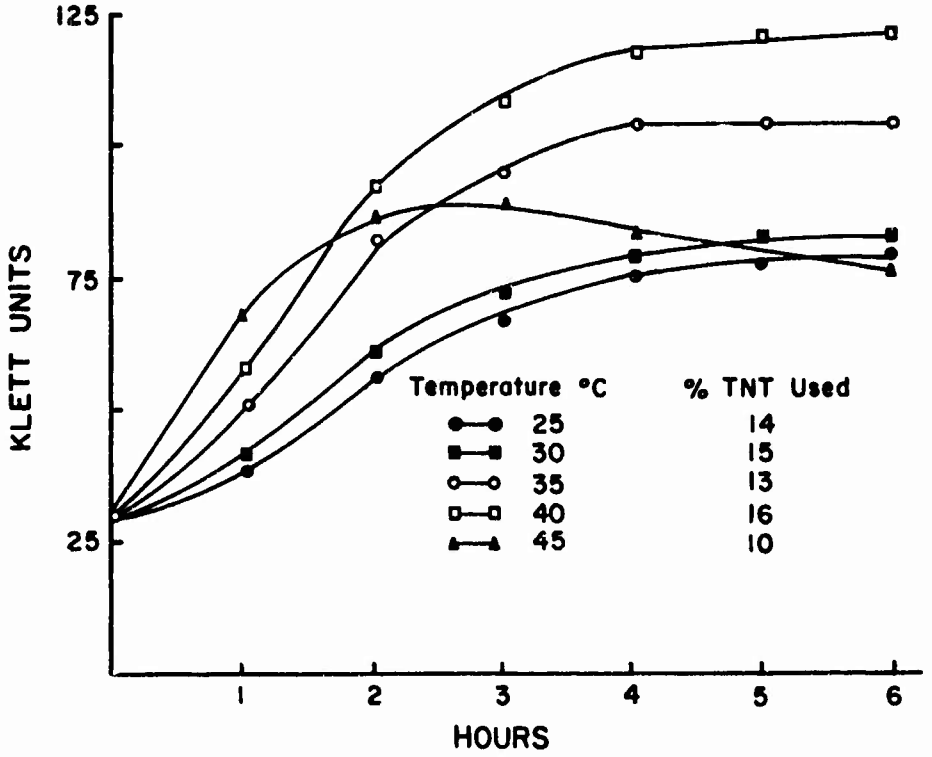

Figure 2. Effect of temperature on growth of IIBX on TNT and percent degradation.

Table 3. Effect of yeast extract concentration on growth of IIBX.

Yeast Extract (µg/ml)	TNT (µg/ml)	Initial Klett Units	Final Klett Units
100	-	21	30
50	-	20	22
10	-	14	11
100	100	40	142
50	100	33	115
10	100	33	57

The effect of temperature on the growth of the organism and disappearance of TNT was investigated using isolate IIBX (Figure 2). Flasks of M-9 + yeast extract (100 µg/ml) + TNT (100 µg/ml) were incubated at 25, 30, 35, 40 and 45C. There is an increased growth rate and final cell density with each 5C increase in the incubation temperature up to 40C. The exponential growth phase is shorter at 45C than at any other temperature and the final cell density is lower. The percentage of the TNT used at each temperature is not significantly different except for possibly 45C, which is the lowest percent utilization.

The relationship between initial TNT concentration in the medium and the percent TNT utilization is inversely related to TNT concentration (Table 4). This inverse effect suggests either TNT inhibition or inhibition by metabolic products.

Table 4. Relationship between substrate concentration and percent utilization.

TNT µg/ml	%TNT Utilized
25	44
50	32
100	28

D. Growth studies with isolate I-2WT and its mutants:

Isolate I-2WT (Full code A7WASN-1) was obtained from the main boiler plant effluent at the University of Rhode Island. This organism was used for growth studies as the wild type and after exposure to UV irradiation for 5, 10, 15 and 20 seconds, the UV irradiated cells were plated on M-9 medium without yeast extract for out growth. Figure 3 shows the growth response of the initial isolate (I-2WT) and the mutant obtained from the 5 second exposure (I-2-5) on M-9 medium supplemented with yeast extract. The 5 and 20 second mutants were grown in M-9 medium without

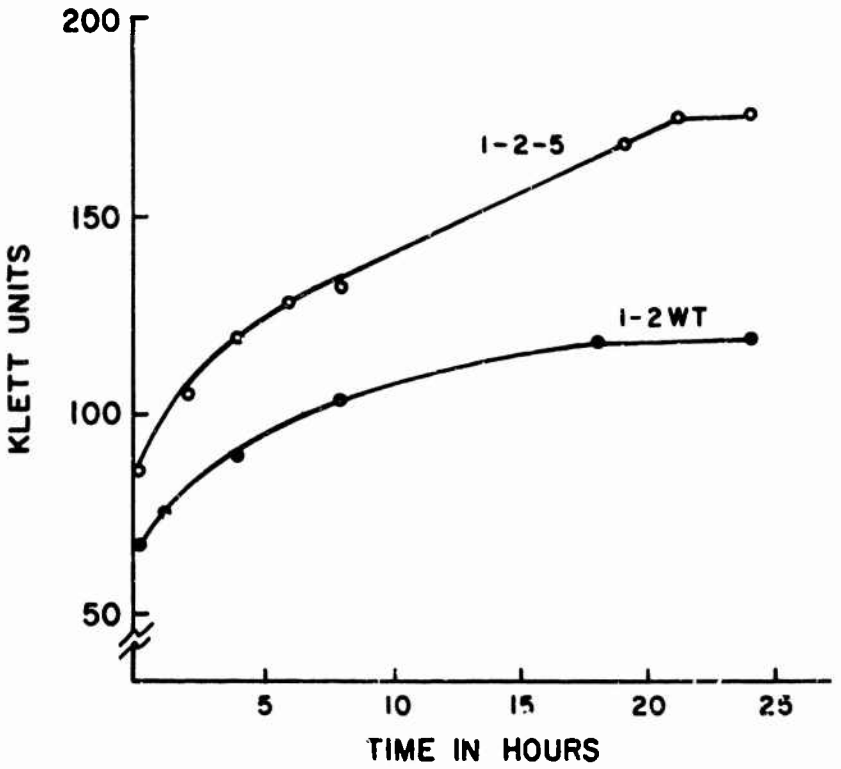

Figure 3. Growth of I-2WT and mutant I-2-5 on TNT.

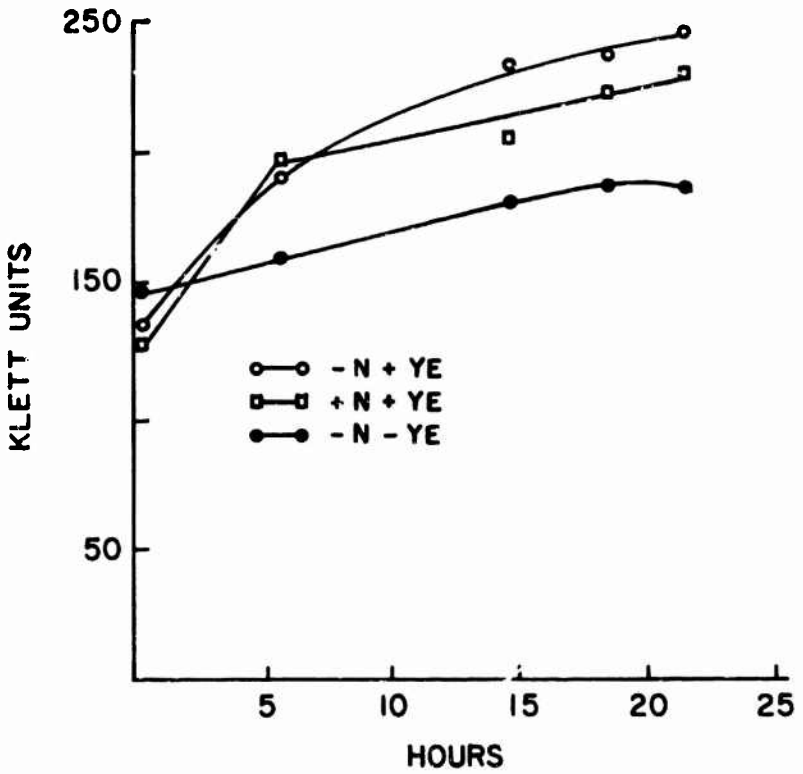

Figure 4. Effect of ammonia and yeast extract on growth of I-2-5 on TNT.

yeast extract supplement. The growth response of the 10 second mutant was essentially the same as the 5 second mutant and the 15 second mutant was like that of the 20 second mutant. The growth of the 5 second mutant (I-2-5) was faster than the wild type or 20 second mutant and reached a greater cell density.

Organism I-2-5 was used to determine the effect of ammonia and yeast extract on the utilization of TNT. Figure 4 shows that in the presence of yeast extract that a slightly better final cell yield is obtained in the absence of ammonia in the medium. Yeast extract is not required for the growth of I-2-5 but has a definite stimulatory effect.

The effect of varying TNT concentration on the growth response and percent TNT utilization is shown in Figure 5. There is a direct relationship between substrate concentration and the amount of growth obtained with I-2-5. Substrate concentration is definitely limiting below 100 µg/ml, and at 25 µg/ml supports very little growth of the organism. In this experiment the TNT served as both the sole source of carbon and nitrogen. With this isolate there is a direct relationship between substrate concentration and percentage of TNT utilized as contrasted to isolate IIBX in which there was an inverse relationship.

E. Other growth and utilization studies:

Nine of the sewage isolates have been screened for growth on TNT (100 µg/ml) in M-9(-N) with 0.01% yeast extract as a supplement. The nature of the growth curves for these isolates is the same as those obtained with isolated I-2WT and IIBX, therefore, we will report only initial and final turbidity readings on these isolates (Table 5).

All nine isolates gave a good growth response in this series. There was no essential difference in the growth rate of any of these isolates with the exception of S-9-SY-UN-1 and S-9-SY-UN-5 which had higher growth rates and the greatest increase in cell density.

Table 5. Growth of sewage isolates on M-9(-N) supplemented with 0.01% yeast extract.

Isolate Number	Klett Reading 0 Hours	Klett Reading 48 Hours
S-9-SY-UN-2	145	315
S-9-SY-UN-6	165	300
S-9-SY-UN-1	135	410
S-9-SY-UN-5	125	420
S-9-SY-UN-3	135	305
S-9-SY-UN-4	195	350
S-9-UY-UN-12	140	300
S-9-UY-UN-DFW	125	280
S-9-UY-UN-5	185	335

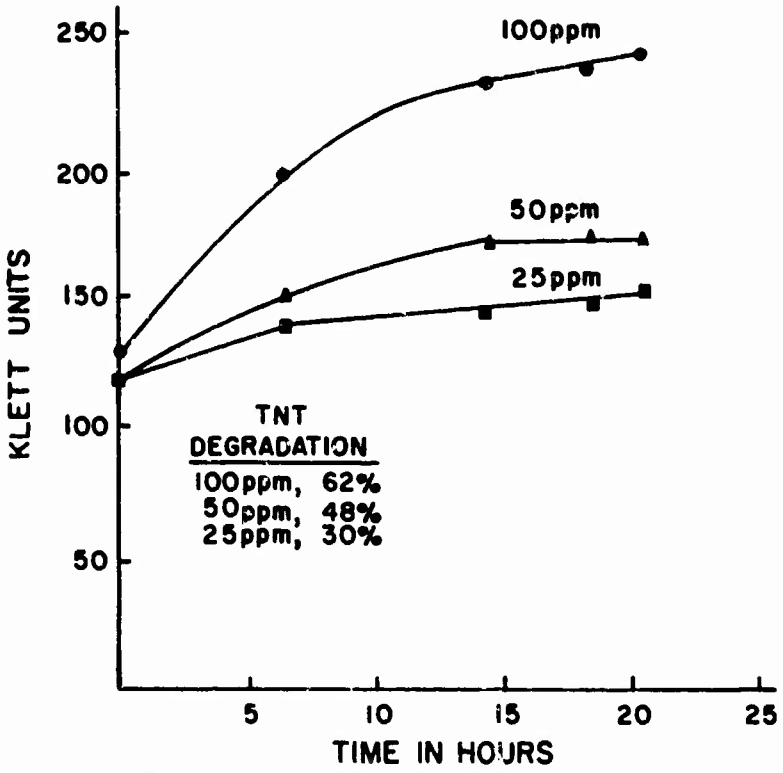

Figure 5. Effect of TNT concentration on growth response of I-2-5 and percent degradation.

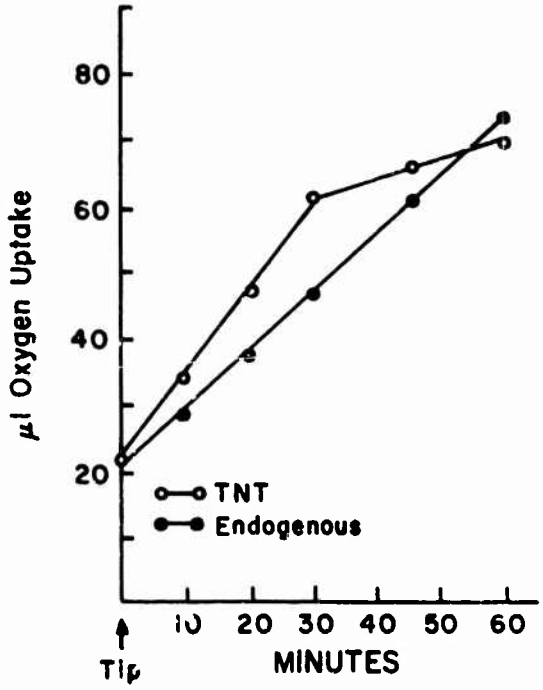

Figure 6. Oxygen uptake by II BX.

F. Metabolic studies:

Results of respirometric studies indicate the oxidation of TNT by isolate IIBX (Figure 6). Oxidation proceeded without a lag following the tip of organisms. During the 30 minutes prior to the break, the O_2 consumption by isolate IIBX on TNT was 34% greater than the endogenous uptake. The oxygen uptake by the endogenous control for the 30 minute period was 25 µliters compared to 38 µliters by TNT. Subtracting the endogenous value indicates a net oxygen uptake of 13 µliters or 0.58 µmoles of oxygen per 0.88 µmoles (200 µg) of TNT in the Warburg flask. It is unlikely that all the TNT was oxidized in this experiment, therefore it is possible that 1 µmole of oxygen is taken up for each µmole of TNT oxidized. This data is not conclusive since the amount of gas exchange is so low that a large margin for error can exist. The amount of carbon dioxide evolved was extremely low and could not be measured accurately in this experiment.

An endogenous repression study was performed by growing the cells on UL-^{14}C glucose to label the cellular reserve materials. The labeled cell suspension was placed in phosphate buffer (0.01M, pH7.0) with and without TNT. Both flasks were swept with air during metabolism to carry any produced carbon dioxide into a methanol-ethanolamine trap. The dpm for the test and control flask were the same (254 and 256) for the initial period of the experiment, indicating no repression of the endogenous metabolism by TNT. At 100 minute of incubation (after the oxygen break in this experiment), there was an observed 35% greater CO_2 evolution in the absence of TNT than in its presence indicating a 35% repression of the endogenous metabolism by the presence of TNT. Since the repression occurs following the oxygen break, it would appear that calculations made prior to the break are valid. It does, however, indicate an effect by TNT or its metabolic products on secondary metabolic events in isolate IIBX.

When (ring-UL-^{14}C) TNT became available, experiments were performed to determine the fate of TNT. Isolates IIBX and I-2WT were grown in flasks of M-9 supplemented with TNT (100 µg/ml) and 0.01% yeast extract (Figure 7). TNT (ring-UL-^{14}C) at 6.4 x 10^6 dpm was added to each flask. The accumulative dpm collected as carbon dioxide are shown in this plot. Table 6 shows the activity obtained in the filtrate after 420 minutes incubation. The recovery of ^{14}C was only 3.8% for isolate IIBX and 5.9% for isolate I-2WT of the initial activity added to the flask. In the experimental protocol, the cells from the reaction flask were recovered on a Millipore membrane and the filtrate activity measured. The cells were then washed with a large volume of water to remove any unmetabolized TNT which might have attached to the cells.

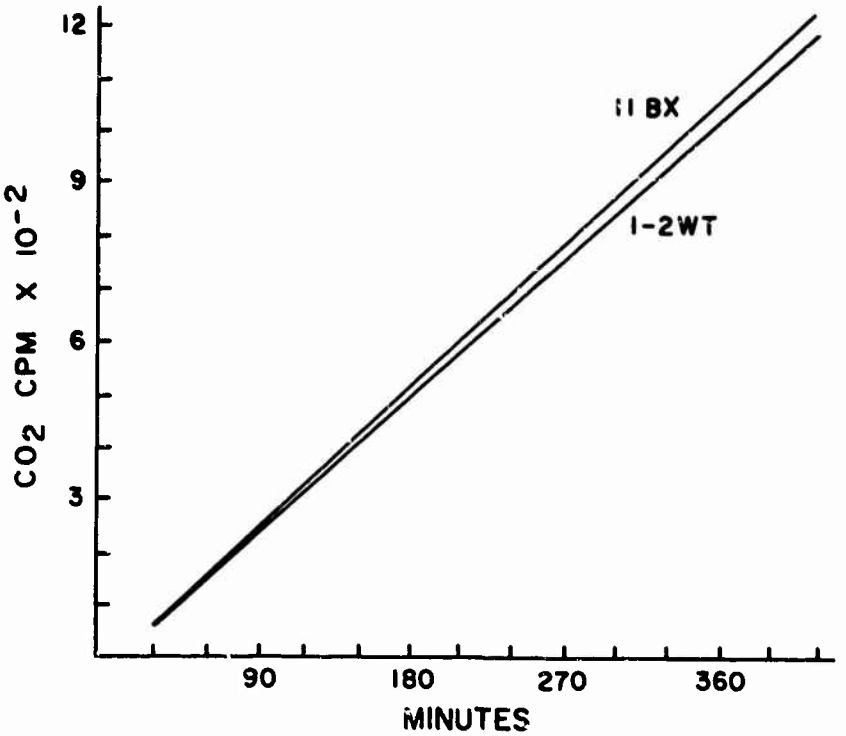

Figure 7. Accumulated dpm as carbon dioxide with IIBX and I-2-5 growing on TNT (ring-UL-^{14}C).

Table 6. TNT (ring-UL-^{14}C) distribution with IIBX and I-2WT growing on TNT in M-9 medium.

Sample	IIBX		I-2WT	
	dpm	% Recovered Activity	dpm	% Recovered Activity
Initial flask	6.4×10^6		6.4×10^6	
Final Filtrate	2.1×10^5	87.0	3.5×10^5	93.0
Cells	3.0×10^4	12.5	2.5×10^4	6.6
Accumulated CO_2	1.2×10^3	0.5	1.2×10^3	0.3
Total Activity Recovered	2.4×10^5	3.8	3.8×10^5	5.9

The measured percent utilization of TNT by isolate IIBX in previous growth systems has varied from 12-28% of an initial concentration of 100 µg/ml. The 13.0% of the recovered label incorporated into the cells and released as carbon dioxide in this experiment by isolate IIBX is, therefore, at the lower range of TNT utilization as shown by the spectrophotometric method. In this experiment flask agitation was not mechanically possible and aeration depended only on the air sweep, thus cutting the aeration efficiency of the system below our normal value. It appears that the ^{14}C values for isolate I-2WT are low when compared to utilization measurements by the spectrophotometer. This ^{14}C data indicates about 7% utilization versus the 20-27% utilization by our standard method.

The experiment was repeated with two important modifications. The reaction was run for 22 hours in a shaker water bath, which slightly increased the aeration efficiency of the system and NH_4Cl was not used in the medium. The results are reported in Table 7.

Table 7. Percentage of labeled TNT metabolized by IIBX and I-2WT in ammonia-free medium.

Sample	IIBX		I-2WT	
	dpm	%	dpm	%
Initial Activity	1.3×10^6	-	1.3×10^6	-
Cells	2.1×10^4	9.6	2.6×10^4	6.1
Filtrate	2.0×10^5	89.3	3.9×10^5	93.1
CO_2	2.7×10^3	1.2	3.5×10^3	.8
Total Recovered	2.2×10^5	17.4	4.2×10^5	32.6

The percentage of the initial activity recovered in this experiment was low

(17.4% for IIBX and 32.6% for I-2WT). We know from previous experiments that neither isolate can utilize the majority of the TNT in these batch culture systems. Also, the aeration efficiency of the ^{14}C apparatus is much lower than our normal growth system, therefore, we expect a reduction in TNT utilization. The low percentage recovery of ^{14}C activity is due to the loss of TNT which has been absorbed to the cells during incubation and its removal by the vigorous wash of the cells. For this reason it is logical to calculate percentages based on recovered activity rather than initial activity. Current and future experiments will include counts of the wash water to substantiate the fate of the "lost" activity.

The data in Table 7 shows 9.6% uptake of recovered label by the IIBX cells and 6.1% uptake by the cells of I-2WT grown on the labeled TNT. The trapped carbon dioxide contained 1.2% and 0.8% of the recovered activity. Since the TNT was labeled only in the ring, any labeled carbon dioxide must result from cleavage of the benzene ring. Thin layer chromatography examination of the labeled TNT does not detect any impurities in this preparation. This experiment is not definitive proof of ring metabolism but is suggestive of ring fission and further experiments are planned to confirm this activity. Warburg respirometric studies confirmed the low level of carbon dioxide production by these isolates. Emphasis will be placed on the study of the cellular products as an indication of TNT utilization rather than carbon dioxide evolution because of the low amounts of carbon dioxide released in this system.

These organisms are required to obtain their carbon for growth only from TNT which is present at low substrate levels (100 µg/ml). It is likely, therefore, that most of the metabolized carbon is used for the synthesis of cellular material. It is expected that in the course of metabolism, decarboxylations do occur in the overall metabolic process, which should result in a net production of measurable amounts of carbon dioxide. The low levels of carbon dioxide detected with these isolates suggested that heterotrophic carbon dioxide fixation might be an important phenomenon in this system (6).

To test this hypothesis, $NaHC^{14}O_3$ was added to a culture of organism I-2-5 growing on unlabeled TNT in the absence of NH_4Cl. The inocula cells were grown on p-hydroxybenzoate and nutrient agar. The flask contained an excess of bicarbonate (20 mg) carrying 1.59×10^6 dpm of ^{14}C activity. Carbon dioxide produced from the metabolism of TNT equilibrates with the C^{14} bicarbonate added as an exogenous supply in these experiments. Dilution experiments based on specific activity would be required to determine the percentage contribution of each source of carbon dioxide. The TNT concentration was 100 µg/ml or a total flask content of 5 mg. The cells were collected at the end of the growth period (22 hours) and counted for ^{14}C incorporation (Table 8). The cells grown in the presence of the labeled carbonate incorporated 2.0% (2.2×10^4 dpm) of the activity or 0.4 mg of carbonate. The flask contained 5 mg of TNT (22 µmoles) and showed the fixation of 9 µmoles of carbon dioxide or 41% heterotrophic carbon dioxide fixation. Previous experiments have indicated that organism I-2-5 only utilized 60-70% of the TNT at a substrate concentration of 100 µg/ml suggesting that the actual percentage of heterotrophic

carbon dioxide fixation is much higher. There was no significant difference in the values obtained in this experiment based upon the substrate used to grow the inoculum cells.

Table 8. Heterotrophic carbon dioxide fixation by I-2-5.

Sample	p-OH Benzoate Grown		Nutrient Agar Grown	
	dpm	% Recovery	dpm	% Recovery
Initial $NaHCO_3$	1.6×10^6	-	1.6×10^6	-
Cells	3.2×10^4	2.0	3.5×10^4	2.0
Filtrate	8.5×10^5	53.0	1.0×10^6	63.0
Total Recovery	8.9×10^5	56.0	1.1×10^6	69.0
Cells based on Recovery	-	4.0	-	3.0

Cells grown on TNT agar demonstrate a definite reddish-brown colony color that is not apparent in nutrient agar grown cells of any of the isolates. Also, in liquid medium this same reddish-brown color is apparent in the filtrate. The color is not identical to the red color which developes in TNT media exposed to light. The ^{14}C-TNT experiments all indicate a significant incorporation of labeled carbon from TNT into the cell. The ^{14}C incorporated from TNT could be as cellular components, accumulated TNT, partial degradation products of TNT or metabolic transformation products of TNT. In an effort to determine the nature of the incorporated ^{14}C and also the reddish-brown color described above, organism I-2-5 was grown on carrier TNT with ring-UL-^{14}C-TNT. After growth, the cells were collected and counted for activity. The dpm from a 25 ml aliquot of cells was 9840. The cells from the remaining 25 ml of the culture were lysed with 5 ml of concentrated H_2SO_4. The lysed cells were extracted twice with 25 ml of petroleum ether, the extracts combined, evaporated to dryness and resuspended in 1.0 ml of petroleum ether. The concentrated extract was spotted (200 lambda) onto prewashed silica gel TLC plates and developed in Benzene: Hexane:Acetone (50:50:10), dried, and sprayed with ethylenediamine. A purple spot was obtained at an R.f of 0.80 which corresponded to 2,2',6,6'-tetranitro-4,4'-azoxytoluene in our known mixture. In the killed cell control, the same spot was found as well as a second spot identified as alpha-TNT. The spots were removed from the TLC plate and extracted into toluene, flor added and counted in the scintillation spectrophotometer. The activities in the two spots of azoxy compound were 4 dpm and the TNT spot was 10 dpm. These low counts are not conclusive but indicate that these components probably have no significance as the cellular incorporated ^{14}C (9840 dpm) from the labeled substrate. Earlier experiments without labeled TNT have indicated the same 2,2',6,6'-tetranitro-4,4'-azoxytoluene component in TNT grown cells and an additional pink spot at an Rf of 0.76 that does not correspond to any compound in our reference mixture.

V. Conclusions

1. Bacteria have been isolated which can utilize 2,4,6-trinitrotoluene (TNT) as a sole source of carbon and nitrogen for growth. All isolates obtained are Gram negative rods. Yeast extract (0.01%) is stimulatory and is required for growth of all isolates. Mutants of isolate I-2WT do not require yeast extract for growth but are stimulated by its addition. Colonies of the various isolates have been obtained on mineral salts medium containing 100 µg/ml of TNT, 0.01% yeast extract and solidified with 1.5% washed agar.

2. Using spectrophotometric analysis of TNT, it has been demonstrated that the various isolates cause a 10-72% disappearance of TNT from the growth medium. The percentage of TNT utilized is related to the initial substrate concentration in the growth medium.

3. Studies with labeled TNT (ring-UL-^{14}C) have shown the incorporation of 6-12% of the label into the cells and the release of 0.8-1.2% of the label as carbon dioxide. Since the TNT is labeled only in the ring, this suggests TNT ring cleavage by the bacteria. It is concluded, therefore, that isolated IIBX and I-2WT may be capable of TNT biodegradation.

4. Studies with $NaHC^{14}O_3$ demonstrate heterotrophic carbon dioxide fixation by organism I-2-5 growing on TNT. This isolate growing on 22 µmoles of TNT fixed 9 µmoles of carbon dioxide.

5. Preliminary experiments suggest that the ^{14}C label from TNT metabolism which is incorporated into the cell is not in the form of nitroaromatic compounds.

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